

Low Endotoxin Recovery (LER) in Drug Products

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Low endotoxin recovery (LER) is a recently observed phenomenon referring to the inability to recover known amounts of endotoxin from specific stored biological drug products (1). Investigators from Genentech have identified two common drug excipients associated with this phenomenon: polysorbate and citrate. The Sigma-Aldrich catalog describes polysorbate 20 as greater than 40% lauric acid and polysorbate 80 as typically 70% oleic acid with both having a balance of fatty acid constituents. The U.S. FDA included a stability screen requirement in their 2012 pyrogen and endotoxin guidance, presumably to address questions raised by the Genentech study that centers on the “Stability of assayable endotoxin content.” The issue has come to the forefront of industry in regard to the performance of the Bacterial Endotoxin Test (BET).

Joseph Chen, PhD, and **Anders Vinther**, PhD, from Genentech, described this phenomenon hypothetically as the formation of a product complex that blocks the ability of factor C (the LAL biosensor) to bind endotoxin. In looking at the various substances involved including unspecified products (presumably protein and/or monoclonal antibodies), polysorbate and citrate, a few different potential mechanisms of action arise as listed below (a-e). Users may consider various cross currents as they collectively monitor stability conditions and consider, via a process of elimination, the LER mechanism of action.

(a) Protein aggregation: Many proteins are known to form complexes with endotoxin and thus mask endotoxin in solution (2). Additionally, monoclonal antibodies (MAbs) are known to form aggregates in aqueous solution, particularly as added surfactants (such as polysorbate) degrade spontaneously (3).

(b) Polysorbates: Polysorbate is used to prevent protein aggregation and loss of drug utility. Per **Edward Maggio**: “Aggregation, which is prevented by the addition of surfactants, and peroxide damage, which

is caused by surfactant-generated peroxides cause an increase in unwanted protein immunogenicity” (4). Protein degradation has been recognized as so severe that some (5) recommend the replacement of polysorbates as stabilizers in drug formulations. Fatty acids are another degradation product of polysorbates. A major coconstituent of polysorbate 20 (up to 25%) and polysorbate 80 (up to 5%) is myristic acid—the key marker in gauging endotoxin content via GC methods. Studies of hydroxymyristic acid as a marker for endotoxin detection date to the ’70s (6,7).

(c) Mild hydrolysis: Endotoxin, biochemically synthesized by bacteria (seven separate enzymatic steps including the use of acyltransferases to add fatty acid acyl groups to the core) (8), is a hardy molecule, removed by washing/rinsing/ binding or destroyed using dry heat (250°C for 30 min, USP). Less severe conditions, however, have also been found to modify the molecule so it’s much less active or inactive, particularly the use of acids or bases with heat. Tirsoaga, et al. (9) found even milder acidic conditions can prompt the reaction in the presence of surfactant (SDS): “Milder hydrolysis conditions such as pH 4.4-4.5 in sodium acetate buffer were shown to be efficient for lipid A liberation and were usually improved by the addition of sodium dodecylsulfate (SDS) when the hydrolysis kinetics were too slow or ineffective.”

Thus, mild acid, a surfactant/ detergent (SDS versus polysorbate) and sodium salt (acetate versus citrate) brought about significant hydrolysis. Mild basic hydrolysis of LPS has also been shown using a 1: 3 dilute solution of NH_4OH at room temperature for 16 hours (10). Changes in endotoxin acyl chain distribution are known to bring about different biosensor responses to endotoxin across the animal kingdom.

(d) Sample hold conditions: Products containing polysorbates may require the absence of oxygen and light to prevent degradation. Singh, et al. suggest that recently introduced ultrarefined polysorbate

80 (>99% oleic acid) may prevent peroxide formation and thus increase the photostability of formulated solutions (11). The high variability of polysorbate fatty acid content for any given formulation is evidenced in USP/EP requirement ranges and as detailed by various vendor COAs.

(e) Immunogenicity and the Pyrogen test: Many may wonder: “What is wrong with a self-depyrogenating solution?” The fear is that endotoxin may be unmasked in the body as positive pyrogen data would suggest. The possibility remains, however, that solutions have been inactivated in terms of endotoxin and the fever in rabbits came from immunogenicity (12) via the therapeutic protein itself. It is not clear if non-spiked solutions were tested side by side with endotoxin spiked solutions to preclude this possibility. The LER issue could turn out to be overstated as the current basis of the differential reaction said to exist between LAL and the pyrogen assay may be based upon borderline pyrogen responses (all the data is not public). The pyrogen test sets called “pyrogenic” would have to be repeated on five more rabbits (only day 0 and day 7 data shown) and have at least four of eight total rabbits with >0.5° C temperature rise to be considered pyrogenic, as per USP <151>.

Explanations that fit the Quality by Design paradigm of increasing product and process knowledge should be explored by characterizing drug products, polysorbates, and degradation constituents in biologics exhibiting the phenomenon. At this point there are many more questions than answers—and answers cannot come without widespread dissemination of details. By understanding the conditions in which LER occurs, BET users can identify situations requiring greater scrutiny as to whether specific drugs are affected by LER.

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[Editor's Note: Low endotoxin recovery will be addressed during a session at PDA's upcoming 8th Annual Global Conference on Pharmaceutical Microbiology. See also the Science Snapshot on p. 20 about the recently released Technical Report No. 3 which covers endotoxin recovery.]

About the Author

Kevin L. Williams, currently at Hospira, has 30 years of experience in the pharmaceutical industry, specializing in endotoxin testing and control. He has written extensively on the subject of LAL technology. 🍷

